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Molecular Phylogeny and Chromosomal Evolution of Japanese *Schoenoplectus* (Cyperaceae), Based on ITS and ETS 1f Sequences

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ITS and ETS 1f sequence data were used to estimate the phylogeny of 13 Japanese *Schoenoplectus* species, and karyomorphological observations were made on 14 species of this genus. Two major clades were identified in the Japanese *Schoenoplectus* molecular phylogenetic tree: (1) one including all species of section *Actaeogeton*, and (2) the another comprising the two sections *Malacogeton* and *Schoenoplectus*. Phylogenetic analysis, including three published species of section *Schoenoplectus*, supported a monophyly of the two major clades. These molecular phylogenetic data also support the intrageneric relationships and morphological characters of genus *Schoenoplectus*, as defined by Smith & Hayasaka (2001). The section *Actaeogeton* clade and the sections *Malacogeton* and *Schoenoplectus* clade showed the same chromosomal evolution; each clade had both high and low chromosome numbers. The high chromosome numbers may arise by polyploidy because chromosome sizes were almost equal in both. Therefore, chromosomal evolution in the genus *Schoenoplectus* may be caused more by polyploidy more than aneuploidy. In our study, the putative natural hybrids, *S.* × *trapezoideus* and *S.* × *uzenensis* were found. The chromosome number of *S.* × *trapezoideus* was $2n = 43$ and *S.* × *uzenensis* was $2n = 58$. These two hybrids had an intermediate chromosome number of both putative parents.

Key words: chromosomal evolution, Cyperaceae, diffuse centromeric chromosome, ITS and ETS 1f phylogeny, *Schoenoplectus*

The genus *Schoenoplectus* (Rchb.) Palla is distributed worldwide, mainly in marsh and wetland habitats, and includes about 77 species (Smith & Hayasaka 2002, Smith 2002). *Schoenoplectus* has been recently accepted as a distinct genus, one of the segregated genera from the polymorphic and polyphyletic genus *Scirpus sensu lato* (Goetghebeur & Simpson 1991, Bruhl 1995, Goetghebeur 1998, Hayasaka & Ohashi 2000, Smith & Hyasaka 2001, 2002). Genus *Schoenoplectus* can be distinguished from closely related genera (*Actinoscirpus*, *Amphiscirpus*, *Blysmus*, *Bolboschoenus*, *Isolepis*, *Oxycaryum*, *Schoenoplectus*, *Scirpus*, *Trichophorum* and *Websteria*) by the following morphological characters: embryo form, stolon presence, tuber presence and form, ligule presence, culm branching, leaf position and structure, spikelet arrangement, floral scale indument, and culm anatomy (Smith & Hayasaka 2001, 2002).

Smith & Hayasaka (2001) recognized four sections, *Actaeogeton*, *Malacogeton*, *Schoenoplectus* and *Supini*, within the genus *Schoenoplectus*. Recently, Pignotti & Mariotti (2004) identified two groups in the genus *Schoenoplectus*, based on the

micromorphology of the fruit surface and pollen size: (1) species of section *Schoenoplectus*, and (2) species of the two sections *Actaeogeton* and *Supini*.

According to the molecular phylogenetic studies of Muasya *et al.* (1998, 2000, 2001), genus *Schoenoplectus* forms a clade with the genera *Actinoscirpus* or *Bolboschoenus*. However, there has been no molecular phylogenetic study of the intrageneric relationships of the genus *Schoenoplectus*.

The chromosome numbers of the genus *Schoenoplectus* have been reported by Hicks (1928), Tanaka (1938, 1948), Otzen (1962), Schuyler (1969, 1972, 1976), Sanyal & Sharma (1972), Rath & Patnaik (1974), Heiser (1979), Iwasaki & Ueki (1979), Löve (1981), Kozhevnikov *et al.* (1986), Subramanian (1988), Bir *et al.* (1991), Hoshino *et al.* (1993), Smith (2002), and Maeda & Uchino (2004). The lowest chromosome number of the genus is $2n$=10 (Schuyler 1969), and the highest number is $2n$=128 (Hicks 1928). Cytological works on Japanese *Schoenoplectus* were carried out by Tanaka (1938, 1948), Iwasaki & Ueki (1979), and Maeda & Uchino (2004), and they observed $2n$=38, 40, 42, 72, 74 and 76 in eight species. Intraspecific aneuploids were $2n$=38, 40 and 42 in *S. lacustris* (Tanaka 1938). The mixoploids were reported as $2n$=32-39 for *S. mucronatus*, $2n$=37-44 for *S. triangulatus* and $2n$=68-76 for *S. gemmifer* (Maeda & Uchino 2004). However, there has been no study on the relationship between chromosomal evolution and phylogeny in the genus *Schoenoplectus*.

DNA sequences are commonly used in phylogenetic analyses and have proven useful in the subfamily Mapanioideae (Simpson *et al.* 2003). In Cyperaceae, plastid *rbcL* gene, *ndhF*, *rps*16, and *trnL* spacers sequence data have been used to estimated higher-level phylogenetic relationships, such as interfamily, intertribal, or suprageneric (Plunkett *et al* 1995, Muasya *et al.* 1998, 2000, 2001, Yen & Olmstead 2000, Roalson *et al.* 2001, Simpson *et al.* 2003). The nrITS sequence data have been used to estimated intrageneric relationships (*e.g., Carex*: Starr *et al.* 1999, Hendrichs *et al.* 2004a, b; *Eleocharis*: Roalson & Friar 2000, Yano *et al.* 2004). Recently, new sequences, the nrDNA noncoding fragments from the external transcribed spacers 1 (ETS 1f) sequences, were reported to estimate lower-level relationships in sedges (Starr *et al.* 2003). The ETS 1f region within intergenic spacer (IGS) is variable and informative more than ITS region (Starr *et al.* 2003). Furthermore, nuclear ITS and ETS 1f sequences were used successfully by Roalson & Friar (2004) and Starr *et al.* (2004) on the genera *Carex* and *Uncinia*. Chromosomal evolution based on molecular data of the genus *Eleocharis* was also reported by Yano *et al.* (2004). The goal of the present study was to clarify the intrageneric relationships of Japanese *Schoenoplectus* using nuclear ribosomal internal transcribed spacer (ITS) and external transcribed spacer 1 (ETS 1f) sequence data, and to identify the relationship between chromosomal evolution and the molecular phylogeny.

Materials and Methods

Plant Samples

Thirteen species of the genus *Schoenoplectus* were sampled for the phylogenetic analyses (Table 1). Samples of living plants were collected from different localities in Japan, and dried specimens were selected from the Herbariums of Okayama University of Science (OKAY) and Tohoku University (TUS). Three sections, *Actaeogeton*, *Malacogeton*, and *Schoenoplectus*, of the genus *Schoenoplectus*, as defined by Smith & Hayasaka (2001) were studied. Section *Supini* could not be included in this study due to the unavailability of plant material. To test for monophyly of the genus *Schoenoplectus*, we chose *Bulbostylis barbata*, *Fimbristylis ovata*, and *Trichophorum alpinum* as outgroups.

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from 0.1 g fresh-frozen material or 0.03 g dried specimens, using a Nucleon Phytopure plant and fungal DNA extraction kit (Amersham Inc.). Amplification of the nrDNA internal transcribed spacers (ITS) region and the nrDNA external transcribed spacers 1 (ETS 1f) region were performed by polymerase chain reaction (PCR) with Taq polymerase (TaKaRa Inc.). Reactions were performed using a DNA thermal cycler (GeneAmp PCR System 2400, Perkin Elmer Inc.) for the ITS region, following the protocol of Hsiao *et al.* (1994), and for the ETS 1f region, following the protocol of Starr *et al.* (2003). The entire ITS-region was amplified with primers ITSL (5′-TCGTAACAAGGTTTCCGTAGGTG-3′; Hsiao *et al.* 1994) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White *et al.* 1990). ETS 1f sequences were amplified using primers ETS 1f (5′-CTGTGGCGTCGCATGAGTTG-3′; Starr *et al.* 2003) and 18S-R (5′-AGACAAGCATATGACTACTGGCAGG-3′; Starr *et al.* 2003). PCR products were electrophoresed on 1.4% agarose gels to confirm a single product, and purified using a Qiaquick PCR purification Kit (Qiagen Inc.).

Cycle sequencing reactions were performed using the purified PCR products and a BigDye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems Inc.). For ITS sequencing, one of the amplification primers, ITSL and ITS4, or one of the internal sequencing primers ITS2C (5′-GCTACGTTCTTCATCGATCG-3′; Yano *et al.* 2004) and ITS3C (5′-GCATCGATGAAGAACGTAGC-3′; Yano *et al.* 2004) was used, and for ETS 1f sequencing, ETS 1f and 18S-R was used. The products were resolved by capillary electrophoresis using an ABI PRISM 310 automated DNA sequencer. Sequences were assembled and edited using Sequencing Analysis version 3.0 (Perkin Elmer Inc.).

Data analyses

The data matrices of the ITS and ETS 1f sequences, including those of the 13 Japanese *Schoenoplectus* species and three outgroups, were used for the phylogenetic analyses. The ITS data matrices of three North American *Schoenoplectus* from Roalson & Friar (2000) were also analyzed.

Sequences were aligned using Clustal W (Thompson *et al.* 1994) and then adjusted manually as necessary. Phylogenetic analyses were performed following the modified methods of Tsubota *et al.* (2002, 2003), Oguri *et al.* (2003), and Yano *et al.* (2004). Gaps were treated as missing data and not used as characters. Phylogenetic trees were constructed using the methods of maximum-parsimony (MP) (Fitch 1971) and maximum-likelihood (ML) (Felsenstein 1981) for each region. MP trees were constructed using PAUPRat (Sikes & Lewis 2001), which is a tool to implement the Parsimony Ratchet searches (Nixon 1999) with PAUP*, over PAUP* 4.0b10 (Swofford 2003), and DNAPARS in PHYLIP 3.573c (Felsenstein 1980-2001). ML trees were constructed by NucML in MOLPHY version 2.3b3 (Adachi & Hasegawa 1996), fastDNAml version 1.2.2 (Olsen *et al.* 1994), DNAML in PHYLIP 3.573c, and PUZZLE version 5.0 (Strimmer & Haeseler 1996). For ML analyses, the HKY85 model (Hasegawa *et al.* 1985) was used as the estimate model. A transition/transversion (Ts/Tv) parameter of 2.39/1.91 (ITS/ETS 1f) was used based on the calculations of PUZZLE.

A tree comparison with the ML criteria by NucML was carried out to evaluate the topology of trees obtained from the two methods (ML and MP), and the program CONSEL 0.1f (Shimodaira & Hasegawa 2001) was also used to calculate the *p*-values of confidence for the bifurcating candidate topologies using test procedures. The ITS tree, including the published ITS data of 3 species in the genus *Schoenoplectus*, was also constructed using the similar method.

The obtained highest log-likelihood ITS tree and ETS 1f tree were applied by TotalML in MOLPHY to evaluate the resulting trees. Using NucML,

TABLE 1. Taxa used in this study and the classification of the genus *Schoenoplectus* according to Smith & Hayasaka (2001). Genbank accession numbers listed for ITS and ETS 1f data. Vouchers are deposited in OKAY and TUS.

Taxon	Locality and Voucher	Chromosome number; 2n (n)	Previous report (2n)	Accession number ITS	ETS 1f
Schoenoplectus					
Sect. Actaeogeton					
S. gemmifer C. Sato, T.Maeda & Uchino			74 (Maeda &		
Japan, Shizuoka Pref., Hamamatsu; *Kitamura 19554* (OKAY)		76	Uchino 2004)	AB206257	AB206271
Japan, Shizuoka Pref., Hamamatsu; *Kitamura 19557* (OKAY)		76			
S. hondoensis (Ohwi) Soják					
Japan, Niigata Pref., Yuzawa; *Sasaki 19618* (OKAY)		38		AB206258	AB206272
S. hotarui (Ohwi) Holub			44 (Iwasaki &		
Japan, Shizuoka Pref., Hamakita; *Hoshino et al. 13744* (OKAY)		42	Ueki 1979)		
Japan, Okayama Pref., Maniwa; *Furuta & Ikeda 15907* (OKAY)		42			
Japan, Okayama Pref., Maniwa; *Furuta et al. 15425* (OKAY)		42 (21_{II})			
Japan, Hiroshima Pref., Fukuyama; *Hoshino 16017* (OKAY)		42			
Japan, Okayama Pref., Okayama; *Sata et al. 16039, 16040* (OKAY)		42			
Japan, Okayama Pref., Sohjya; *Katayama 17521* (OKAY)				AB180720	AB206273
Japan, Miyagi Pref., Watari; *Hayasaka & Yano 18997* (OKAY)		44			
Japan, Hokkaido Pref., Tomakomai; *Hoshino et al. 19269* (OKAY)		44		AB206259	AB206274
S. juncoides (Roxb.) Palla			74 (Iwasaki &		
Japan, Okayama Pref., Sohjya; *Hoshino et al. 17539* (OKAY)			Ueki 1979)	AB206260	AB206275
Japan, Yamaguchi Pref., Yanai; *Kaita 18181* (OKAY)		74			
Japan, Okinawa Pref., Kunigami; *Yokota 19387* (OKAY)		74			
Japan, Gunma Pref., Agatsuma; *Hoshino et al. 19519* (OKAY)		74			
S. komarovii (Roshev.) Soják					
Japan, Yamanashi Pref., Minami-tsuru; *Katsuyama & Yano 18213* (OKAY)				AB206261	AB206276
Japan, Hokkaido Pref., Tomakomai; *Hoshino et al. 19279* (OKAY)		38 (19_{II})			
S. lineolatus (Franch. & Sav.) T. Koyama			42, ca. 60		
Japan, Okayama Pref., Sohjya; *Katayama 17573* (OKAY)		74	(Tanaka 1948,		
Japan, Yamanashi Pref., Minami-tsuru; *Hoshino et al. 17864* (OKAY)		74	Kozhevnikov *et al.* 1986)		
Japan, Yamanashi Pref., Minami-tsuru; *Katsuyama & Yano 18216* (OKAY)		74 (37_{II})		AB206262	AB206277
S. mucronatus (L.) Palla			38 (Maeda &		
Japan, Okayama Pref., Okayama; *Furuta et al. 16013, 16014* (OKAY)		38	Uchino 2004)		
Japan, Okayama Pref., Tamano; *Hirahara & Yano 18136* (OKAY)		38		AB206263	AB206278
S. multisetus Hayasaka & C. Sato					
Japan, Kumamoto Pref., Kumamoto; *Sato s. n.* (TUS)				AB206264	AB206279
Japan, Okayama Pref., Okayama; *Hirahara et al. 19661* (OKAY)		70			

TABLE 1. Continued.

Taxon	Locality and Voucher	Chromosome number; 2n (n)	Previous report (2n)	Accession number ITS	ETS 1f
Schoenoplectus					
Sect. Actaeogeton					
S. triangulatus (Roxb.) Soják			42 (Tanaka 1948, Maeda & Uchino 2004)		
	Japan, Okayama Pref., Okayama; *Furuta et al. 15708* (OKAY)	42			
	Japan, Hiroshima Pref., Fukuyama; *Hoshino 17567* (OKAY)			AB206265	AB206280
	Japan, Shizuoka Pref., Hikisa; *Kitamura 19548* (OKAY)	42			
	Japan, Shizuoka Pref., Hamamatsu; *Kitamura 19556* (OKAY)	42			
S. wallichii (Ness) T. Koyama			72 (Iwasaki & Ueki 1979)		
	Japan, Kagawa Pref., Nakatado; *Hirahara et al. 18332* (OKAY)			AB206266	AB206281
S. × *trapezoideus* (Koidz.) Hayasaka & H. Ohashi		43			
	Japan, Miyagi Pref., Sendai; *Hayasaka 2483* (TUS)				
S. × *uzenensis* (T. Koyama) Hayasaka & H. Ohashi		58			
	Japan, Fukushima Pref., Soma; *Hayasaka 2309* (TUS)				
Sect. Malacogeton					
S. nipponicus (Makino) Soják			76 (Tanaka 1948)		
	Japan, Hokkaido Pref., Tomakomai; *Hoshino et al. 19265-19267* (OKAY)	74		AB206267	AB206282
Sect. Schoenoplectus					
S. tabernaemontani (C. C. Gmel.) Palla			40, 42, 44 (Otzen 1962, Schuyler 1976, Hoshino *et al.* 1993, Smith 2002.)		
	Japan, Okayama Pref., Bizen; *Katayama 17660* (OKAY)			AB206268	AB206283
	Japan, Kagoshima Pref., Nase; *Tsusaka 18968* (OKAY)	42			
S. triqueter (L.) Palla			40, 41, 42, 44 (Tanaka 1948, Otzen 1962, Bir *et al.* 1991, Hoshino *et al.* 1993, Smith 2002.)		
	Japan, Okayama Pref., Maniwa; *Furuta & Ikeda 13172* (OKAY)	42			
	Japan, Okayama Pref., Maniwa; *Furuta et al. 15415* (OKAY)			AB206269	AB206284
Outgroups					
Bulbostylis					
B. barbata Kunth					
	Japan, Okayama Pref., Okayama; *Hoshino et al. 13779* (OKAY)			AB180718	AB206285
Fimbristylis					
F. ovata (Burm. f.) Kern					
	Japan, Kanagawa Pref., Miura; *Yano 18218* (OKAY)			AB180719	AB206286
Trichophorum					
T. alpinum (L) Pers.					
	Japan, Hokkaido Pref., Nakagawa; *Sato 13260* (OKAY)			AB206270	AB206287

local bootstrap probabilities (LBPs; %) (Adachi & Hasegawa 1996) were applied to each best topology.

Chromosome observations

Fourteen species of the genus *Schoenoplectus* collected from Japan were used for karyomorphological observations. Voucher specimens are listed in Table 1. Somatic chromosomes of the genus *Schoenoplectus* were observed in the meristematic cells of root tips or shoots. The root tips or shoots were pretreated in 0.002 M 8-hydroxyquinoline for 5 hr at 16 °C or for 1 hr at 23 °C and 15 hr at 4 °C. They were fixed in acetic alcohol (1: 3) for over 16 hr at −20 °C or for 1.5 hr at 23 °C, stained by Feulgen's nuclear reaction, macerated in a mixture of 2% pectinase and 2% cellulase for 1 hr at 37 °C, restained in 1% aceto-orcein, and squashed. Meiotic chromosomes were also observed in pollen mother cells (PMCs). Spikelets were fixed in acetic alcohol (1: 3) for over 6 hr at −20 °C. The anthers were stained in 1% aceto-orcein and then squashed.

Results

Sequence analyses

The ITS sequences of the 16 species, including the three outgroups, ranged in length from 375 to 462 bp. Aligned sequences were 514 bp, of which 342 sites were used for the phylogenetic analyses. The data matrix contained 273 variable sites, of which 188 were potentially phylogenetically informative.

The ETS 1f sequences of the 16 species, including the three outgroups, ranged in length from 551 to 651 bp. Aligned sequences were 736 bp, of which 514 sites were used for the phylogenetic analyses. The data matrix contained 395 variable sites, of which 279 were potentially phylogenetically informative.

In phylogenetic study using nrITS/ETS

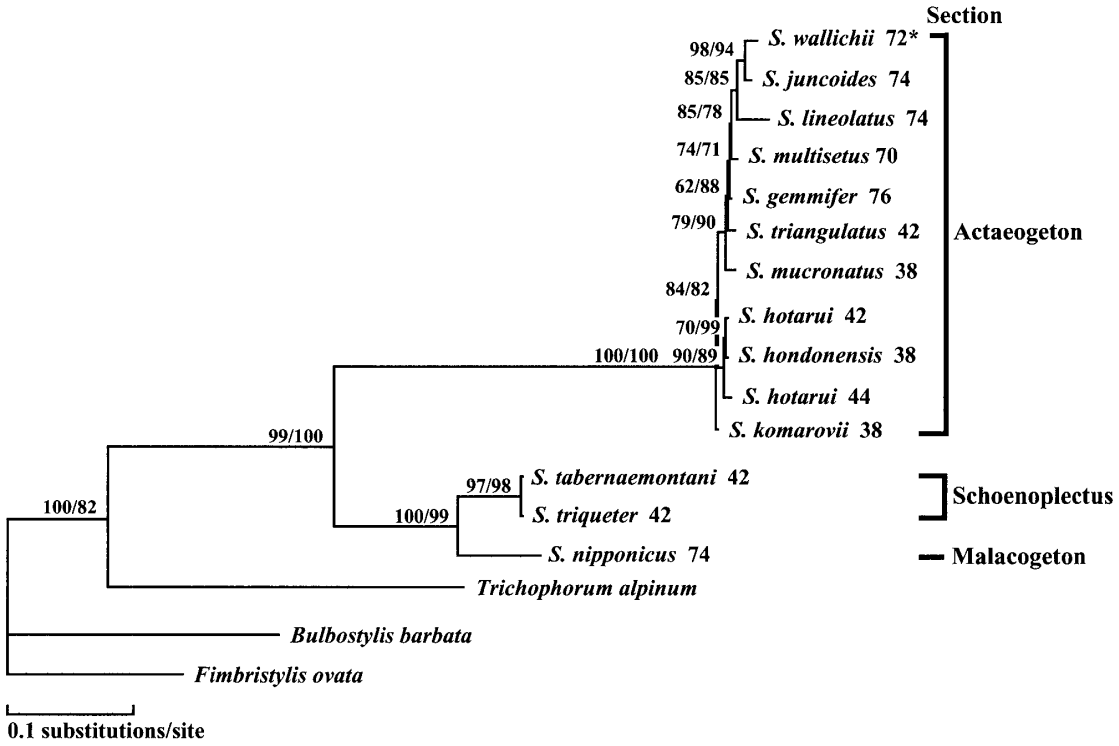

FIG. 1. Total ML tree of the ITS and ETS 1f sequences in Japanese *Schoenoplectus* (HKY85 model; ln *L*=-4738.1 by NucML). Local bootstrap probabilities (LBP; %) more than 50% are shown above branches (ITS/ETS 1f). The intrageneric classification by Smith & Hayasaka (2001) is shown on the right. The numbers after each taxon are chromosome numbers (2*n*), and * showed previously reported.

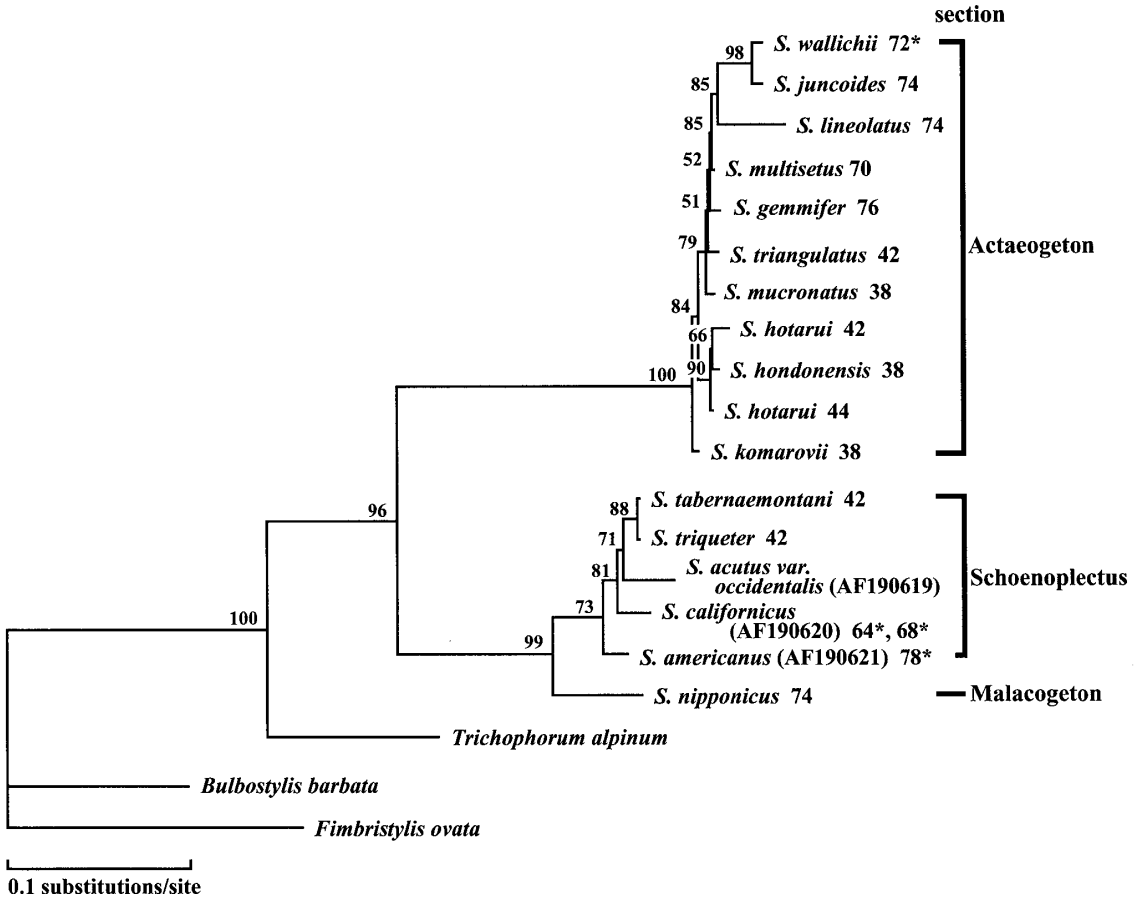

FIG. 2. The best NucML tree by ITS data including the 3 published species of the genus *Schoenoplectus* (HKY85 model; $\alpha/\beta = 5.315$; ln $L = -1817.16 \pm 73.67$). Local bootstrap probabilities (LBP; %) more than 50% are shown above branches. The intrageneric classifications by Smith & Hayasaka (2001) are shown on the right. The numbers after each taxon are chromosome numbers ($2n$), and * showed previously reported. GenBank accession numbers are also to the right of each taxon name.

sequence data, it is necessary to consider the presence of ITS/ETS intraspecific polymorphisms, paralogous copies of ITS/ETS in the genome, and pseudogene copies of the nrDNA repeat (Roalson & Friar 2004). However, Roalson & Friar (2004) reported that paralogous and pseudogene copies of ITS/ETS are not having a major influence on the topology of the ITS/ ETS phylogeny in *Carex*. In this study, ITS/ETS sequences were not cloned, because polymorphic sequences which show paralogous copies of the nrDNA were not found.

Phylogenetic analyses

For phylogenetic analyses, a total of nine (ITS) and ten (ETS 1f) non-overlapping topologies were obtained from the MP and ML methods. More detailed topologies were searched for through the obtained trees using log-likelihood measure (NucML and CONSEL 0.1f). The highest log-likelihood tree for ITS and ETS 1f was obtained, as in Fig. 1. Phylogenetic analyses of the Japanese species revealed two major clades in the genus *Schoenoplectus* (Fig. 1): (1) all sampled species of section *Actaeogeton* (100% LBP value), and (2) sections *Malacogeton* and *Schoenoplectus* (100/99% support in Total ML tree).

Aligned sequences of the ITS region for 19 species, including 3 North American and 13 Japanese *Schoenoplectus* species and 3 outgroups, were 514 bp, of which 332 sites were used for the phylogenetic analyses. The data matrix contained 277 variable sites, of which 170 were potentially

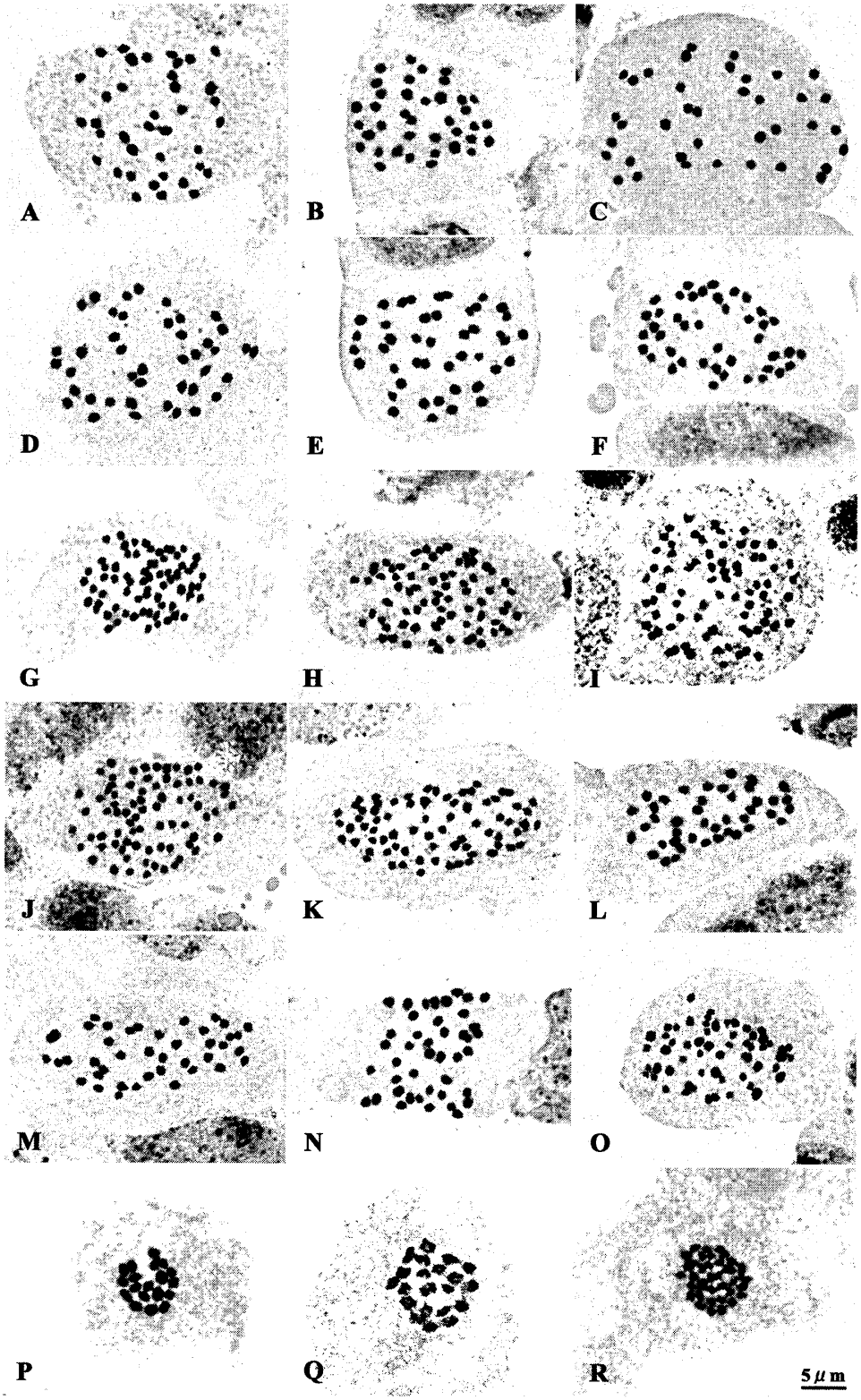

FIG. 3. Photomicrographs of somatic metaphase chromosomes (A-O) and meiotic metaphase I chromosomes (P-R) of the genus *Schoenoplectus*. A-J, N-R, Section *Actaeogeton*. K, Section *Malacogeton*. L, M, Section *Schoenoplectus*. A, *Schoenoplectus hondoensis* (2*n*=38). B, *S. komarovii* (2*n*=38). C, *S. mucronatus* (2*n*=38). D, E, *S. hotarui* (2*n*=42, 44). F, *S. triangulatus* (2*n*=42). G, *S. multisetus* (2*n*=70). H, *S. juncoides* (2*n*=74). I, *S. lineolatus* (2*n*=74). J, *S. gemmifer* (2*n*=76). K, *S. nipponicus* (2*n*=74). L, *S. tabernaemontani* (2*n*=42). M, *S. triqueter* (2*n*=42). N, *S.* × *trapezoideus* (2*n*=43). O, *S.* × *uzenensis* (2*n*=58). P, *S. hotarui* (2*n*=42=21_{II}). Q, *S. komarovii* (2*n*=38=19_{II}). R, *S. lineolatus* (2*n*=74=37_{II}).

phylogenetically informative. The ML tree, obtained from phylogenetic analyses using the 19 species, also showed two major clades in the genus *Schoenoplectus* (Fig. 2): (1) all sampled species of section *Actaeogeton* with 100% bootstrap support, and (2) the rest of *Schoenoplectus* with 99% bootstrap support.

Chromosome observations

Cytological studies were conducted in 14 species. Chromosome numbers were observed to be $2n$=38 for *Schoenoplectus mucronatus*, $2n$=42 for *S. tabernaemontani*, $2n$=42 for *S. triangulatus*, $2n$=42 for *S. triqueter*, $2n$=44 for *S. hotarui*, and $2n$=74 for *S. juncoides*, which confirm previous reports (Tanaka 1948, Iwasaki & Ueki 1979, Hoshino *et al.* 1993, Smith 2002, Maeda & Uchino 2004) (Table 1). The chromosome numbers of $2n$=38 for *S. hondoensis*, $2n$=38=19II for *S. komarovii*, and $2n$=70 for *S. multisetus* were determined for the first time in this study. New chromosome numbers observed in this study were $2n=42=21_{II}$ for *S. hotarui*, $2n=74=37_{II}$ for *S. lineolatus*, $2n$=74 for *S. nipponicus*, and $2n$=76 for *S. gemmifer* (Fig. 3, Table 1). Intraspecific aneuploids were found to be $2n$=42 and 44 in *S. hotarui*. Two major groups were recognized by the following karyomorphological observations: (1) low chromosome numbers, including species with $2n=38=19_{II}$, $2n=42=21_{II}$ and 44, and where somatic chromosomes ranged from 0.7 to 1.3 μm in length and meiotic metaphase I ranged from 1.8 to 2.8 μm in length, and (2) high chromosome numbers, including species with $2n$=70, $2n=74=37_{II}$ and 76, and where somatic chromosomes ranged from 0.5 to 1.1 μm in length, and meiotic metaphase I chromosomes ranged from 1.2 to 2.2 μm in length (Fig. 3).

Maeda & Uchino (2004) reported a continuous series of mixoploids in root-tip cells: $2n$=32-39 for *Schoenoplectus mucronatus*, $2n$=37-44 for *S. triangulatus*, and $2n$=68-76 for *S. gemmifer*. In our results, *S. mucronatus* was $2n$=38 in 39 cells of two individuals from two localities, *S. triangulatus* was $2n$=42 in 51 cells of three individuals from three localities, and *S. gemmifer* was $2n$=76 in 28 cells of two individuals from two localities. In this study, mixoploids were not found in these three species of *Schoenoplectus*.

In our study, two kinds of putative natural hybrids, *Schoenoplectus* × *trapezoideus* and *S.* × *uzenensis*, were found. The chromosome number of *S.* × *trapezoideus* was $2n$=43 and *S.* × *uzenensis* was $2n$=58. These chromosome numbers were determined for the first time in this study. The 43 metaphase chromosomes of *S.* × *trapezoideus* ranged from 0.7 to 1.3 μm in length, and 58 metaphase chromosomes of *S.* × *uzenensis* ranged from 0.5 to 1.3 μm in length.

Discussion

Intrageneric relationships and morphological characters

Smith & Hayasaka (2001) recognized four sections (*Actaeogeton*, *Malacogeton*, *Schoenoplectus* and *Supini*) in the genus *Schoenoplectus*. In their classification, the ornament and color of achenes, and the type of floral scale apices, are used as the major characters that separate sections *Actaeogeton* and *Supini* from the other two sections. In species of sections *Actaeogeton* and *Supini* the achenes are rugulose and blackish when ripe and the floral scale apices are entire, but in species of sections *Malacogeton* and *Schoenoplectus* the achenes are smooth and yellow-dark brown when ripe and the floral scale apices are emarginate to bifid or very obscure. The veins and apices of floral scale apices, rhizomes and tubers are important characters to separate sections *Schoenoplectus* and *Malacogeton*. In section *Malacogeton*, floral scales have 2-10 prominent veins and are very obscure apices, the rhizomes are weak or soft and tubers are present, whereas in section *Schoenoplectus* the veins are absent and apices are emarginate to deeply bifid, rhi-

zomes are strong and tubers are absent. From the ML tree (Figs. 1, 2), sections *Actaeogeton* and *Schoenoplectus* are monophyletic with a high statistical confidence (100, 97/98, and 73% LBP). Section *Malacogeton*, including only one species in this study, clearly divided from section *Schoenoplectus*. These molecular phylogenetic data support intrageneric relationships in genus *Schoenoplectus*, as defined by Smith & Hayasaka (2001). Moreover, our study reinforces that the ornament and color of achenes, the type of floral scale, rhizomes and tubers are important morphological characters.

Pignotti & Mariotti (2004) identified two groups in genus *Schoenoplectus* based on the micromorphology of fruit surface and pollen size: (1) species of section *Schoenoplectus*, and (2) species comprising sections *Actaeogeton* and *Supini* (sensu Smith & Hayasaka 2001). The cells of the pericarp in species of section *Schoenoplectus* are roughly hexagonal and isodiametrical, and pollen grains in these species are longer than 40 μm, but the cell of the pericarp in species comprising sections *Actaeogeton* and *Supini* are extremely narrow, longitudinally oblong, arranged transversely, and pollen grains are shorter than 40 μm. Our phylogenetic trees show strong support for monophyly of sections *Actaeogeton* and *Schoenoplectus* (Figs. 1, 2). Therefore, micromorphology of fruit surface and pollen size may be also useful characters for the classification of the genus *Schoenoplectus*.

Karyotype and chromosomal evolution

The genus *Schoenoplectus* can be classified into two major groups based on karyotype (Fig. 3): (1) species with high chromosome numbers ($2n$=70, 74 and 76), and (2) species with low chromosome numbers ($2n$=38, 42 and 44). When these karyomorphological data and previous reported chromosome numbers are superimposed on the ML tree (Figs. 1, 2), section *Actaeogeton* had both high chromosome numbers ($2n$=70, 72, 74 and 76) and low chromosome numbers ($2n$=38, 42 and 44). Sections *Malacogeton* and *Schoenoplectus* also had both high chromosome numbers ($2n$=64, 68, 74 and 78) and low chromosome numbers ($2n$=42).

Intraspecific aneuploids were reported in *Schoenoplectus lacustris* ($2n$=38, 40 and 42), *S. tabernaemontani* ($2n$=40, 42 and 44), and *S. triqueter* ($2n$=40, 41, 42 and 44) (Tanaka 1938, 1948, Otzen 1962, Schuyler 1976, Bir *et al.* 1991, Hoshino *et al.* 1993, Smith 2002). In this study, we founded intraspecific aneuploids in *S. hotarui* ($2n$=42, 44), and in *S. gemmifer* and *S. nipponicus* ($2n$=74, 76) (Fig. 3, Table 1). The origin of intraspecific aneuploids is thought to be by chromosome mutation (Yano *et al.* 2004). Hoshino *et al.* (2000) reported that aneuploids in Cyperaceae are thought to arise by chromosome fusion or fragmentation. Practically, intraspecific aneuploids are common in members of Cyperaceae plants with diffuse centromeric chromosomes. In this study, intraspecific aneuploids may also arise by chromosome fusion or fragmentation, because genus *Schoenoplectus* possesses diffuse centromeric chromosomes.

Schoenoplectus × *trapezoideus* and *S.* × *uzenensis* are putative natural interspecific hybrids based on morphological data (Koyama 1958, Hayasaka & Ohashi 2000). *Schoenoplectus* × *trapezoideus* is considered the natural hybridization or introgression of *S. hotarui* and *S. triangulatus*, and *S.* × *uzenensis* is considered the natural hybridization or introgression of *S. triangulatus* and *S. lineolatus*. In our study, *S.* × *trapezoideus* showed $2n$=43, and the putative parents of this hybrid were *S. hotarui* ($2n$=44) and *S. triangulatus* ($2n$=42). *S.* × *uzenensis* showed $2n$=58, and the putative parents were *S. triangulatus* ($2n$=42) and *S. lineolatus* ($2n$=74). These two hybrids had intermediate chromosome numbers of both putative parents. Our cytological results supported that these two hybrids were arisen by the natural hybridization or introgression. Further analysis of the meiotic configurations is needed to determine the parents of these nat-

ural hybrids.

Roalson *et al.* (2001) speculated that the ancestor of the Cariceae had a moderate to high chromosome number, and that low chromosome numbers might represent a derived condition in *Carex* based on a molecular phylogeny of tribe Cariceae. They hypothesized that agmatoploids might result from chromosome fusion at least as often as from chromosome fission. Yano *et al.* (2004) reported chromosome diversity caused by both aneuploidization and polyploidization in *Eleocharis* that possess diffuse centromeric chromosomes. In *Carex*, Hoshino (1981) reported when the chromosome number increases by aneuploidy, both lengths and width tend to become small.

In our current study, two major clades of *Schoenoplectus*, (1) section *Actaeogeton*, (2) sections *Malacogeton* and *Schoenoplectus*, had both high and low chromosome numbers. The high chromosome numbers might have arisen by polyploidy because chromosome sizes were almost equal in both. Therefore, chromosomal evolution in the genus *Schoenoplectus* may be caused more by polyploidy more than aneuploidy.

Future studies should include examining more samples and plastid sequences, and combining analyses of nuclear and plastid sequence data would be useful to understand fully the phylogenetics and chromosome evolution of the genus *Schoenoplectus*.

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